

TITLE: MIMOTOPES AND ANTI-MIMOTOPES OF
HUMAN PLATELET GLYCOPROTEIN Ib/IX

INVENTOR(S): Jonathan L. Miller
Vicki A. Lyle

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Case	Age	Sex	Occupation	Duration of illness	Site of lesion	Pathological changes	Microscopic findings	Diagnosis
1	45	M	Farmer	10 years	Brain	Chronic	Microscopic	Chronic
2	55	F	Housewife	5 years	Brain	Chronic	Microscopic	Chronic
3	65	M	Teacher	15 years	Brain	Chronic	Microscopic	Chronic
4	75	F	Retiree	20 years	Brain	Chronic	Microscopic	Chronic
5	85	M	Farmer	25 years	Brain	Chronic	Microscopic	Chronic
6	95	F	Housewife	30 years	Brain	Chronic	Microscopic	Chronic
7	105	M	Farmer	35 years	Brain	Chronic	Microscopic	Chronic
8	115	F	Housewife	40 years	Brain	Chronic	Microscopic	Chronic
9	125	M	Farmer	45 years	Brain	Chronic	Microscopic	Chronic
10	135	F	Housewife	50 years	Brain	Chronic	Microscopic	Chronic

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epitope for C-34 was recently localized to the extracellular portion of the GPIb alpha chain expressed on the surface of Chinese Hamster Ovary cells (Chambers et al. 1995). The failure of C-34 to bind to denatured GPIb alpha in Western blots (Ward and Berndt 1995; Clemetson and Hugli 1995), or to immunoprecipitate the extracellular region of GPIb alpha removed from platelets under a variety of experimental conditions (Miller et al. 1990) strongly suggests that the epitope recognized by C-34 is highly conformation-dependent. Recently Ward and Berndt have, however, now reported the successful immunoprecipitation by C-34 of a 1•His-Arg•293 amino-terminal fragment of ¹²⁵I-labeled glyocalicin following digestion of the purified molecule by trypsin (Ward and Berndt 1995).

Attempts to define the binding sites for various monoclonal antibodies have led to the development of epitope libraries. Parmley and Smith developed a bacteriophage expression vector that could display foreign epitopes on its surface (Parmley and Smith 1988). This vector could be used to construct large collections of bacteriophage which could include virtually all possible sequences of a short (e.g. six-amino-acid) peptide. They also developed biopanning, which is a method for affinity-purifying phage displaying foreign epitopes using a specific antibody (see Parmley and Smith 1988; Cwirla et al. 1990; Scott and Smith 1990; Christian et al. 1992; Smith and Scott 1993).

After the development of epitope libraries, Smith et al. then suggested that it should be possible to use the bacteriophage expression vector and biopanning technique of Parmley and Smith to identify epitopes from all possible sequences of a given length. This led to the idea of identifying peptide ligands for antibodies by

biopanning epitope libraries, which could then be used in vaccine design, epitope mapping, the identification of genes, and many other applications (Parmley and Smith 1988; Scott 1992).

5 Using epitope libraries and biopanning, researchers searching for epitope sequences found instead peptide sequences which mimicked the epitope, i.e., sequences which did not identify a continuous linear native sequence or necessarily occur at all within a
10 natural protein sequence. These mimicking peptides are called mimotopes. In this manner, mimotopes of various binding sites/proteins have been found. LaRocca et al. (1992) expressed a mimotope of the human breast epithelial mucin tandem repeat in *Escherichia coli*.
15 Balass et al. (1993) identified a hexapeptide that mimics a conformation-dependent binding site of the acetylcholine receptor. Hobart et al. (1993) isolated a mimotope that mimics the C6 epitope (the epitope for the sixth component of complement).

20 The sequences of these mimotopes, by definition, do not identify a continuous linear native sequence or necessarily occur in any way in a naturally-occurring molecule, i.e. a naturally occurring protein. The sequences of the mimotopes merely form a peptide
25 which functionally mimics a binding site on a naturally-occurring protein. For example, the mimotope of Balass et al. (1993) mimics the binding site of the acetylcholine receptor.

 Many of these mimotopes are short peptides.
30 The availability of short peptides which can be readily synthesized in large amounts and which can mimic naturally-occurring sequences (i.e. binding sites) offers great potential application.

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A need continues to exist, therefore, for the elucidation of useful mimotopes.

SUMMARY OF INVENTION

5 This need is met by the mimotopes of the subject invention. The invention thus provides an isolated peptide that functionally mimics a binding site for a monoclonal antibody, the monoclonal antibody recognizing an epitope within the human platelet glycoprotein Ib/IX complex. This isolated peptide is a mimotope. A peptide functionally mimics a binding site for a monoclonal antibody if the monoclonal antibody can bind to the peptide. Preferably, the isolated peptide comprises an amino acid sequence as shown in SEQ ID
10 NO:174: WRXXEY.

The invention further provides an isolated molecule capable of binding to the peptide, which molecule can be an antibody, a second peptide, a carbohydrate, a DNA molecule, an RNA molecule, or any
20 chemically synthesized molecule, for example. This isolated molecule is an anti-mimotope. Anti-mimotopes that bind to a receptor can be used to mediate the functional activity of that receptor. Preferably, the isolated molecule is capable of binding to the isolated
25 peptide described above (the isolated peptide that comprises an amino acid sequence as shown in SEQ ID NO:174). This preferred isolated molecule inhibits ristocetin induced aggregation of platelets and has a three dimensional structure complementary to the three
30 dimensional structure of the isolated peptide.

The invention also provides a method for modulating the adhesion, aggregation, or agglutination of platelets, each of which is dependent on von Willebrand factor interaction with platelets through the

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glycoprotein Ib/IX complex receptor. The methods provide for exposure of platelets to the molecule (anti-mimotope) in order to modulate adhesion, aggregation, or agglutination of the platelets.

5 The invention further provides a method of identifying a molecule that inhibits ristocetin induced aggregation of platelets. The method comprises determining whether a molecule binds to the isolated peptide described above (the isolated peptide that
10 comprises an amino acid sequence as shown in SEQ ID NO:174), and screening a molecule that binds to the peptide to determine whether the screened molecule inhibits ristocetin induced aggregation of platelets.

 The invention further provides an isolated
15 peptide capable of binding to monoclonal antibody C-34, as well as an isolated molecule capable of binding to such peptide. Also provided is a method for modulating the adhesion, aggregation, or agglutination of platelets by exposing the platelets to the molecule (anti-
20 mimotope).

 In a preferred embodiment, the isolated peptide capable of binding to monoclonal antibody C-34 includes an amino acid sequence corresponding to SEQ ID NO:38: WNWRYREYV.

25 The invention still further provides an isolated peptide capable of binding to monoclonal antibody SZ-2, as well as an isolated molecule capable of binding to such peptide. Also provided is a method for modulating the adhesion, aggregation, or agglutination of
30 platelets by exposing the platelets to the molecule (anti-mimotope).

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These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in
5 conjunction with the accompanying drawings in which:

Fig. 2 illustrates the inhibition of ristocetin-induced aggregation of platelets by 20 $\mu\text{g/ml}$ of monoclonal antibody C-34;

Fig. 4 illustrates the partial neutralization of the inhibition of ristocetin-induced aggregation of platelets by 20 $\mu\text{g/ml}$ of mab C-34 in the presence of 0.27 μM of the synthetic peptide mimotope having SEQ ID NO: 1:
20 AAWNRYREYV;

Fig. 5 illustrates the partial neutralization of the inhibition of ristocetin-induced aggregation of platelets by 20 $\mu\text{g/ml}$ of mab C-34 in the presence of 0.55 μM of the synthetic peptide mimotope having SEQ ID NO: 1:

25 AWWRYREYV;

Fig. 6 illustrates the partial neutralization of the inhibition of ristocetin-induced aggregation of platelets by 20 $\mu\text{g/ml}$ of mab C-34 in the presence of 1.1 μM of the synthetic peptide mimotope having SEQ ID NO: 1:

30 AWWNRYREYV;

Fig. 7 illustrates the complete neutralization of the inhibition of ristocetin-induced aggregation of platelets by 20 $\mu\text{g/ml}$ of mab C-34 in the presence of 2.3

Fig. 8 illustrates the functional screening of candidate anti-mimotope bacteriophage clones. Following incubation of 150 μ L of the indicated bacteriophage clones with 250 μ L of citrated PRP for 1 hr at 22°C, aggregation was initiated by the addition of 0.8 mg/mL ristocetin under stirring conditions at 37°C;

Figs. 12a-12c are a diagrammatic sketch of mimotopes and anti-mimotopes used to probe the structural relationships in platelet glycoprotein Ib alpha.

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In one preferred embodiment, the monoclonal antibody is designated C-34, and the peptide includes an amino acid sequence selected from the group consisting

25 of:

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      SEQ ID NO:1:      AAWNRYREYV
      SEQ ID NO:2:      KWNWRNKKYV
      SEQ ID NO:3:      LSTWRYFEYV
30    SEQ ID NO:4:      YLGWRYSEYV
      SEQ ID NO:5:      TQMWRAREYL
      SEQ ID NO:6:      WRQREYWDPV
      SEQ ID NO:7:      EGSWRYRKGG
      SEQ ID NO:8:      GYHWRNWEY

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SEQ ID NO:9: KGFLWRARNW ✓
SEQ ID NO:10: MNWKHWRARH ✓
SEQ ID NO:11: FKWREWRGKL ✓
SEQ ID NO:12: PDRQVRLWVR ✓
5 SEQ ID NO:13: RVLRHWHHPRT ✓
SEQ ID NO:14: GRRVWMLNHG ✓
SEQ ID NO:15: KKGRHHVTRV ✓
SEQ ID NO:16: GGVCKCWQCL ✓
SEQ ID NO:17: FSHSYGSAIR ✓
10 SEQ ID NO:18: MHGHRRPGLA ✓
SEQ ID NO:19: MSKKPHLGLR ✓
SEQ ID NO:20: TMWVELYSLK ✓
SEQ ID NO:21: FVDPGRAGRG ✓
SEQ ID NO:23: FRCCVFSCCLLS ✓
15 SEQ ID NO:24: GFRCLVSLGGCF ✓
SEQ ID NO:25: YSLWGLPVGDIV ✓
SEQ ID NO:26: LPLLWFNGAGFF ✓
SEQ ID NO:27: VWGLFRGLENGS ✓
SEQ ID NO:28: SLWRQWRGLFVV ✓
20 SEQ ID NO:29: TLSLFGGRDKGF ✓
SEQ ID NO:30: IGPAVSCLEFRVC ✓
SEQ ID NO:31: MSLFPLSFCRLI ✓
SEQ ID NO:32: ALFSSVWGDVTL ✓
SEQ ID NO:33: GWFGPFWVRGSG ✓
25 SEQ ID NO:34: FWVSVGGVEGVV ✓
SEQ ID NO:35: LGAFGGAGFLWR ✓
SEQ ID NO:36: CRGIVFLFVGWL ✓
SEQ ID NO:37: FWLVKGAGAWRF ✓
SEQ ID NO:39: QVRLWARAGAGQ ✓
30 SEQ ID NO:40: GLAVTFGSVLEG ✓
SEQ ID NO:41: VRWMCVIRLGVR ✓
SEQ ID NO:42: RLWGPGVSRPVL ✓
SEQ ID NO:43: CGSSLFRGPRCP ✓
SEQ ID NO:44: LGISSLFLQLR ✓

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SEQ ID NO:45: TWGWDGVSYLFL ✓
SEQ ID NO:46: TRSLFDDFVSLR ✓
SEQ ID NO:47: CYASLFRSRLCA ✓
SEQ ID NO:48: DGSVRVVWVRLL ✓
5 SEQ ID NO:49: LSGFPVALVRFA ✓
SEQ ID NO:50: LGGGLLVGSVFP ✓
SEQ ID NO:51: VWARGVFRDRFF ✓
SEQ ID NO:52: TGLLAGPVWRWT ✓
SEQ ID NO:53: WLGGIFSCLVCG ✓
10 SEQ ID NO:54: WFLRDVGC GSCL ✓
SEQ ID NO:55: SRCGVFTWC SRS ✓
SEQ ID NO:56: RCLVGYRCWGGV ✓
SEQ ID NO:57: GFRCLVMGGGCA ✓
SEQ ID NO:58: CGFDLVCARLFG ✓
15 SEQ ID NO:59: DSGVRWFFGFLG ✓
SEQ ID NO:60: ILDGCFFLGRCP ✓
SEQ ID NO:61: CVRWLV SAGCSG ✓
SEQ ID NO:62: CVGCWLVC DVLL ✓
SEQ ID NO:63: CLFVFAAGFACG ✓
20 SEQ ID NO:64: SCALFGSCFGIS ✓
SEQ ID NO:65: CWGGVGVCGLLV ✓
SEQ ID NO:66: KRAWWKQKWV ✓
SEQ ID NO:67: CVGGVASRCGVL ✓
SEQ ID NO:68: SGAVLAGPFGVW ✓
25 SEQ ID NO:69: CRAFDRVGV CVW ✓
SEQ ID NO:70: RCLVGYVVG VVW ✓
SEQ ID NO:71: VCLVYRSVDCWA ✓
SEQ ID NO:72: WRV FVFTCVVWA ✓
SEQ ID NO:73: LWREWRGLFAVL ✓
30 SEQ ID NO:74: SGAVLAGPLWRL ✓
SEQ ID NO:75: FVV RGGTFLFVR ✓
SEQ ID NO:77: TGLLAGPVWRWT ✓
SEQ ID NO:78: DSGVRWFFGFLG ✓
SEQ ID NO:79: CAWHRLSFCGLV ✓

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SEQ ID NO:80: CFGSALVLAVLA and

SEQ ID NO:81: WFDMSGGEWGGL.

Preferably, the peptide includes an amino acid
5 sequence corresponding to consensus sequence SEQ ID NO:
38: WNWRYREYV.

In a presently preferred embodiment, the
isolated peptide comprises an amino acid sequence as
shown in SEQ ID NO:174: WRXXEY. This consensus sequence
10 is derived from the epitope mapping studies of mab C-34
as discussed below (see series of cloned sequences
included in alignment form). Amino acid residue number 3
of SEQ ID NO:174 is preferably selected from the group
consisting of alanine, asparagine, glutamine, and
15 tyrosine. Amino acid residue number 4 of SEQ ID NO:174
is preferably selected from the group consisting of
arginine, phenylalanine, serine, and tryptophan.

Each of these peptides, represented by SEQ ID
NOS 1 to 21, 23-37, 39-75, 77-81, and 174 mimics the
20 binding site within GPIb/IX for mab C-34. Mab C-34 thus
binds to each of these peptides. However, the sequences
of each of these peptides do not identify a continuous
linear native sequence or necessarily occur at all within
the sequence of any chain (i.e. GPIb alpha, GPIb beta,
25 GPIX) of the GPIb/IX complex, thus the peptides are
mimicking the mab C-34 binding site and are therefore
mimotopes. The peptide of the subject invention also
includes fragments of the above exemplified peptides
which retain the ability to functionally mimic the
30 binding site for a monoclonal antibody, such as C-34.
The peptide having an amino acid sequence corresponding
to SEQ ID NO:38 is an example of such a fragment, being a
fragment of the peptide which includes the amino acid
sequence corresponding to SEQ ID NO:1.

In another embodiment, the monoclonal antibody is designated SZ-2, and the peptide includes an amino acid sequence selected from the group consisting of:

5 SEQ ID NO:83: WHWRSSWKSG
SEQ ID NO:84: HRPLSWKGRA
SEQ ID NO:85: WHRRPMSWYS
SEQ ID NO:86: ARIKIWKPRW
SEQ ID NO:87: KRGWHWKS LH
10 SEQ ID NO:88: KKSWWVRMPR
SEQ ID NO:89: AKSWRYWRMP
SEQ ID NO:90: KRWKVYHRWP
SEQ ID NO:91: LHRWKQSPRT
SEQ ID NO:92: LIRWKPHGWR
15 SEQ ID NO:93: QKKFFSRWKH
SEQ ID NO:76: KWWVPRHRVW
SEQ ID NO:82: RSKWWVHRHS
SEQ ID NO:109: RWWHWVHRET
SEQ ID NO:110: KRWLWWANPR
20 SEQ ID NO:111: RHLWWGGRMK
SEQ ID NO:112: RLWPQHRGHR
SEQ ID NO:113: KRWHIRPTIR
SEQ ID NO:114: KRFKTHVHGR
SEQ ID NO:115: TKRFBKRRHFL
25 SEQ ID NO:116: AKWHWHTRGR
SEQ ID NO:117: WHRHWGGFRI
SEQ ID NO:118: WHRNKPTWHS
SEQ ID NO:119: WHRAGVRAKV
SEQ ID NO:120: FKRFWHTGHR
30 SEQ ID NO:121: MMAWHARVAR
SEQ ID NO:122: WIWHRPIKVK
SEQ ID NO:123: WHRTLPRKRGH
SEQ ID NO:124: VKHFRWRPVA
SEQ ID NO:125: KRHWRFQLSN

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SEQ ID NO:126: KRHRLASMAP
SEQ ID NO:127: WRWRWRGBVLR
SEQ ID NO:128: RLHAHHARHR
SEQ ID NO:129: RWGAKHRVRV
5 SEQ ID NO:130: AMGWRPVKHR
SEQ ID NO:131: KWRWRMHQHY
SEQ ID NO:132: WLSKLGHRHA
SEQ ID NO:133: KHCSIHTRLR
SEQ ID NO:134: GSAERMSEGH
10 SEQ ID NO:135: FPLWNVLTMT
SEQ ID NO:136: SFAGVGWFALLG
SEQ ID NO:137: CDLWVCFLDGGG
SEQ ID NO:138: LVARFPPPYGGV
SEQ ID NO:139: SIVWLTRPKG
15 SEQ ID NO:140: CRYRALNGVL
SEQ ID NO:141: ALTSRTWARQ
SEQ ID NO:142: TRYMLSRQSN
SEQ ID NO:143: AMREARITVK
SEQ ID NO:144: WRRHVPLRIL
20 SEQ ID NO:145: FHRWNRPMVT
SEQ ID NO:146: HRYKKTPVPM
SEQ ID NO:147: WLHVKRRPVV
SEQ ID NO:148: WVRHKHPIVP
SEQ ID NO:149: LSMRRRQFQS
25 SEQ ID NO:150: FHWRDKWRTG
SEQ ID NO:151: RMRRPGITVK
SEQ ID NO:152: GHRWNRPMVT
SEQ ID NO:153: WHRHTPKRIP
SEQ ID NO:154: WHWQSRPAL
30 SEQ ID NO:155: KRTWWHYIRP and
SEQ ID NO:156: KRWRHSLPAS.

Each of these peptides, represented by SEQ ID
NOs 83-93, 76, 82, and 109-156, mimics the binding site

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within GPIb/IX for mab SZ-2. Mab SZ-2 thus binds to each of these peptides, which are referred to as mimotopes. The peptide of the subject invention also includes fragments of the above exemplified peptides which retain the ability to functionally mimic the binding site for monoclonal antibody SZ-2.

As used herein, a "peptide" refers to an amino acid sequence of three to one hundred amino acids, and therefore an isolated peptide that comprises an amino acid sequence is not intended to cover amino acid sequences of greater than 100 amino acids. Preferably, the peptides of the subject invention (whether they be mimotope or anti-mimotope peptides) are less than 50 amino acids in length, and more preferably the peptides are five to 20 amino acids in length or 20-40 amino acids in length.

The peptides described herein can contain any naturally-occurring or non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide is maintained. The choice of including an (L)- or a (D)-amino acid in the peptides of the present invention depends, in part, on the desired characteristics of the peptide. For example, the incorporation of one or more (D)-amino acids can confer increased stability on the peptide and can allow a peptide to remain active in the body for an extended period of time. The incorporation of one or more (D)-amino acids can also increase or decrease the pharmacological activity of the peptide.

The peptides may also be cyclized, since cyclization may provide the peptides of the present invention with superior properties over their linear counterparts.

In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for traditional peptide bonds and backbones. An example of one such modification is the reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such as those disclosed in Wann et al., JOC, 46:257 (1981) and Raucher et al., Tetrahedron. Lett., 21:14061 (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangement between functional groups.

In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for traditional peptide bonds and backbones. An example of one such modification is the reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such as those disclosed in Wann et al., JOC, 46:257 (1981) and Raucher et al., Tetrahedron. Lett., 21:14061 (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangement between functional groups.

One skilled in the art, using the above sequences or formulae, can easily synthesize the peptides of this invention. Standard procedures for preparing synthetic peptides are well known in the art. The novel peptides can be synthesized using: the solid phase peptide synthesis (SPPS) method of Merrifield (J. Am. Chem. Soc., 85:2149 (1964)) or modifications of SPPS; or, the peptides can be synthesized using standard solution methods well known in the art (see, for example, Bodanzsky, M., Principles of Peptide Synthesis, 2nd revised ed., Springer-Verlag (1988 and 1993)). Alternatively, simultaneous multiple peptide synthesis (SMPS) techniques well known in the art can be used. Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, Calif.) or using the manual peptide synthesis technique described by Houghten, Proc. Natl. Acad. Sci., USA 82:5131 (1985).

According to the subject invention, the monoclonal antibody (whose binding site is mimicked by the peptide of the invention, i.e. C-34 or SZ-2)

recognizes an epitope within the human glycoprotein Ib/IX complex.

The invention also provides an isolated molecule capable of binding to the peptide. This isolated molecule is called an anti-mimotope. The anti-mimotope molecule can be any suitable molecule, such as, for example, an antibody, a second peptide, a carbohydrate, a DNA molecule, an RNA molecule, or a chemically synthesized molecule. Such peptides, proteins, or other biological, synthetic, or semi-synthetic molecules that are capable of binding to the mimotope can be identified by: raising antibodies against the mimotope; selecting from bacteriophage, chemical, hybridoma cell, or other types of libraries, cells, or chemical syntheses that might produce a set or subset of molecules having high affinity for the mimotope sequence; or designing molecules intended to have a high affinity for the mimotope sequences using computer-assisted or other theoretical approaches. Suitable anti-mimotopes can also be developed using *in vitro* evolution of nucleic acids capable of binding to the peptide mimotope (see Joyce 1994).

In one embodiment, the anti-mimotope of the subject invention constitutes a peptide which includes an amino acid sequence selected from the group consisting of:

SEQ ID NO:94: RHVAWWRQGV
SEQ ID NO:95: AKHRWWRRPV
30 SEQ ID NO:96: KHFMRRHRHGV
SEQ ID NO:97: AGLNHWWKHK
SEQ ID NO:98: RRSTWHWWHA
SEQ ID NO:99: VAKWRHWNRQ
SEQ ID NO:157: AYGVRHLGLS

SEQ ID NO:158: KKWGQHRQRS
SEQ ID NO:159: WRWMHWMPHA
SEQ ID NO:160: WHWLARHRTV
SEQ ID NO:161: RHRHRGFQPR
5 SEQ ID NO:162: RGWRWHKYWQ
SEQ ID NO:163: KRHAWMK SRL
SEQ ID NO:164: LLLVGGSELT
SEQ ID NO:165: KKVWMFSYNE
SEQ ID NO:166: LSCRCRAFV
10 SEQ ID NO:167: HEGCEAQDEL
SEQ ID NO:168: SVRHIWFHVK
SEQ ID NO:169: GTWDLWRKGS
SEQ ID NO:170: RWLWPRVHKT
SEQ ID NO:171: HSPFRHVQPR and
15 SEQ ID NO:172: WVRGHHREVR.

These particular anti-mimotope peptides were generated to the mimotope which mimics the binding site for monoclonal antibody C-34.

20 Such anti-mimotopes could serve as anti-thrombotic drugs. For example, the binding of mab C-34 to GPIb/IX inhibits ristocetin-induced aggregation of platelets. The mimotope peptide mimics the binding site in GPIb/IX, and the anti-mimotope molecules bind to the
25 mimotope peptide. Therefore, the anti-mimotopes, which could be peptides, should themselves complement the mimotope peptide. As such, the anti-mimotopes should be capable of binding to the original epitope for mab C-34 or mab SZ-2 within the platelet glycoprotein Ib/IX
30 complex, thereby inducing similar effects as does mab C-34 or mab SZ-2, i.e. the inhibition of ristocetin-induced aggregation of platelets that is dependent upon von Willebrand factor.

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The invention thus provides a method of modulating the adhesion, aggregation, or agglutination of platelets, the method comprising selecting platelets and exposing the platelets to the anti-mimotope molecule of 5 the subject invention. Such exposure affects von Willebrand factor interaction with platelets through the glycoprotein Ib/IX receptor, thereby modulating the adhesion, aggregation, or agglutination of the platelets.

The invention also provides an isolated peptide 10 capable of binding to monoclonal antibody C-34, the peptide including an amino acid sequence selected from the group consisting of:

SEQ ID NO:1: AWNWRYREYV
15 SEQ ID NO:2: KWNWRNKKYV
SEQ ID NO:3: LSTWRYFEYV
SEQ ID NO:4: YLGWRYSEYV
SEQ ID NO:5: TQMWRAREYL
SEQ ID NO:6: WRQREYWDPV
20 SEQ ID NO:7: EGSWRYRKGG
SEQ ID NO:8: GYHWWRNWEY
SEQ ID NO:9: KGFLWRARNW
SEQ ID NO:10: MNWKHWRARH
SEQ ID NO:11: FKWREWRGKL
25 SEQ ID NO:12: PDRQVRLWVR
SEQ ID NO:13: RVLRHWHHPRT
SEQ ID NO:14: GRRVWMLNHG
SEQ ID NO:15: KKGRHHVTRV
SEQ ID NO:16: GGVCKCWQCL
30 SEQ ID NO:17: FSHSYGSAIR
SEQ ID NO:18: MHGHRRPGLA
SEQ ID NO:19: MSKKPHLGLR
SEQ ID NO:20: TMWVELYSLK
SEQ ID NO:21: FVDPGRAGRG

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	SEQ ID NO:23:	FRCCVFSCCLLS
	SEQ ID NO:24:	GFRCLVSLGGCF
	SEQ ID NO:25:	YSLWGLPVGDVV
	SEQ ID NO:26:	LPLLWFNGAGFF
5	SEQ ID NO:27:	VWGLFRGLENGS
	SEQ ID NO:28:	SLWRQWRGLFVV
	SEQ ID NO:29:	TLSLFGGRDKGF
	SEQ ID NO:30:	IGPAVSCLFRVC
	SEQ ID NO:31:	MSLFPLSFCRLI
10	SEQ ID NO:32:	ALFSSVWGDVTL
	SEQ ID NO:33:	GWFGPFWVRGSG
	SEQ ID NO:34:	FWVSVGGVEGVV
	SEQ ID NO:35:	LGAFGGAGFLWR
	SEQ ID NO:36:	CRGIVFLFVGWL
15	SEQ ID NO:37:	FWLVKGAGAWRF
	SEQ ID NO:39:	QVRLWARAGAGQ
	SEQ ID NO:40:	GLAVTFGSVLEG
	SEQ ID NO:41:	VRWMCVIRLGVR
	SEQ ID NO:42:	RLWGPGVSRPVL
20	SEQ ID NO:43:	CGSSLFRGPRCP
	SEQ ID NO:44:	LGISSLFLQLR
	SEQ ID NO:45:	TWGWGDVSYLFL
	SEQ ID NO:46:	TRSLFDDFVSLR
	SEQ ID NO:47:	CYASLFRSRLCA
25	SEQ ID NO:48:	DGSVRVVWVRL
	SEQ ID NO:49:	LSGFPVALVRFA
	SEQ ID NO:50:	LGGGLLVGSVFP
	SEQ ID NO:51:	VWARGVFRDRFF
	SEQ ID NO:52:	TGLLAGPVWRWT
30	SEQ ID NO:53:	WLG GIFSCLVCG
	SEQ ID NO:54:	WFLRDVGC GSCL
	SEQ ID NO:55:	SRCGVFTWCSRS
	SEQ ID NO:56:	RCLVGYRCWGGV
	SEQ ID NO:57:	GFRCLVMGGGCA

25

30 ID NO:1.

The invention also provides an isolated molecule capable of binding to the above peptides, also known as an anti-mimotope. Suitable molecules include an

antibody, another peptide, a DNA or RNA molecule, a carbohydrate, or a chemically synthesized molecule.

Preferably, the anti-mimotope is an isolated molecule capable of binding to an isolated peptide,
5 wherein the isolated peptide comprises an amino acid sequence as shown in SEQ ID NO:174. This isolated molecule inhibits ristocetin induced aggregation of platelets and has a three dimensional structure complementary to the three dimensional structure of the
10 isolated peptide (comprising an amino acid sequence as shown in SEQ ID NO:174). The concept of "complementary" is illustrated in Fig. 12a-12c.

The invention provides a method of modulating the adhesion, aggregation, or agglutination of platelets,
15 the method comprising selecting platelets and exposing the platelets to the anti-mimotope molecule. Such exposure affects von Willebrand factor interaction with platelets through the glycoprotein Ib/IX receptor, thereby modulating the adhesion, aggregation, or
20 agglutination of the platelets.

In one preferred embodiment, the invention provides an isolated peptide capable of binding to monoclonal antibody C-34 and including an amino acid sequence corresponding to SEQ ID NO:38: WNWRYREYV.

25 The invention further provides an isolated peptide capable of binding to monoclonal antibody SZ-2, the peptide including an amino acid sequence selected from the group consisting of:

30 SEQ ID NO:83: WHWRSSWKSG
SEQ ID NO:84: HRPLSWKGRA
SEQ ID NO:85: WHRRPMSWYS
SEQ ID NO:86: ARIKIWKPRW
SEQ ID NO:87: KRGWHWKS LH

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SEQ ID NO:88: KKSWWVRMPR
SEQ ID NO:89: AKSWRYWRMP
SEQ ID NO:90: KRWKVYHRWP
SEQ ID NO:91: LHRWKQSPRT
5 SEQ ID NO:92: LIRWKPHGWR
SEQ ID NO:93: QKKFFSRWKH
SEQ ID NO:76: KWWVPRHRVW
SEQ ID NO:82: RSKWWVHRHS
SEQ ID NO:109: RWWHWVHRET
10 SEQ ID NO:110: KRWLWWANPR
SEQ ID NO:111: RHLWWGGRMK
SEQ ID NO:112: RLWPQHRGHR
SEQ ID NO:113: KRWHIRPTIR
SEQ ID NO:114: KRFKTHVHGR
15 SEQ ID NO:115: TKRFKHRHFL
SEQ ID NO:116: AKWHWHTRGR
SEQ ID NO:117: WHRHWWGGFRI
SEQ ID NO:118: WHRNKPTWHS
SEQ ID NO:119: WHRAGVRAKV
20 SEQ ID NO:120: FKRFWHTGHR
SEQ ID NO:121: MMAWHARVAR
SEQ ID NO:122: WIWHRPIKVK
SEQ ID NO:123: WHRTLPKRGH
SEQ ID NO:124: VKHFRWRPVA
25 SEQ ID NO:125: KRHWRFQLSN
SEQ ID NO:126: KRHRLASMAP
SEQ ID NO:127: WRWRWRGVLR
SEQ ID NO:128: RLHAHHARHR
SEQ ID NO:129: RWGAKHRVRV
30 SEQ ID NO:130: AMGWRPVKHR
SEQ ID NO:131: KWRWRMHQHY
SEQ ID NO:132: WLSKLGHRHA
SEQ ID NO:133: KHCSIHTRLR
SEQ ID NO:134: GSAERMSEGH

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SEQ ID NO:135: FPLWNVLTMT
SEQ ID NO:136: SFAGVGWFALLG
SEQ ID NO:137: CDLWVCFLDGGG
SEQ ID NO:138: LVARFPPPYGGV
5 SEQ ID NO:139: SIVWLTRPKG
SEQ ID NO:140: CRYRALNGVL
SEQ ID NO:141: ALTSRTWARQ
SEQ ID NO:142: TRYMLSRQSN
SEQ ID NO:143: AMREARITVK
10 SEQ ID NO:144: WRRHVPLRIL
SEQ ID NO:145: FHRWNRPMVT
SEQ ID NO:146: HRYKKTPVPM
SEQ ID NO:147: WLHVKRRPVV
SEQ ID NO:148: WVRHKHPIVP
15 SEQ ID NO:149: LSMRRRQFQS
SEQ ID NO:150: FHWRDKWRTG
SEQ ID NO:151: RMRRPGITVK
SEQ ID NO:152: GHRWNRPMVT
SEQ ID NO:153: WHRHTPKRIP
20 SEQ ID NO:154: WHWQRSRPAL
SEQ ID NO:155: KRTWWHYIRP and
SEQ ID NO:156: KRWRHSLPAS.

25 Further provided is a fragment of any of the
above peptides wherein the fragment retains the ability
to bind to monoclonal antibody SZ-2. The invention also
provides an isolated molecule capable of binding to the
above peptides (an anti-mimotope), and a method of
30 modulating the adhesion, aggregation or agglutination of
platelets by exposing the platelets to the anti-mimotope
molecule.

The invention further provides a method of
identifying a molecule that inhibits ristocetin induced

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The invention is described in further detail as follows.

As reported by Miller, et al. (1990), platelets from patients with platelet-type von Willebrand disease (PT-vWD) heterozygous for the mutation

230•WKQ(G-V)₂₃₃V•234 in the alpha chain of platelet glycoprotein Ib were used as immunogens for the production of murine mabs. One such mab, C-34, inhibited ristocetin-induced aggregation of patient or normal platelets, but not aggregation induced by other aggregating agents. As demonstrated by crossed-immunoelectrophoresis, mab C-34 recognized an epitope within the GPIb/IX complex. In indirect immunofluorescence studies on fresh platelets, the ratio

of any of four different anti-GPIb mabs to one another was near unity (0.88-1.14) both for normals and for patients. In contrast, the ratio of the binding of mab C-34 to such a mab (AP-1) was 0.31 ± 0.02 (means \pm SE) for normal platelets and significantly increased to 0.54 ± 0.01 for patient platelets ($p < 0.001$). In immunoprecipitations on NP-40 lysates of ^3H -labeled platelets, saturating concentrations of mab C-34 produced much fainter bands than did AS-2 or other anti-GPIb mabs. In contrast to the other anti-GPIb mabs, C-34 did not bind to the purified ^{125}I -labeled glyocalicin fragment of GPIb or to the glyocalicin derivative identified by crossed-immunoelectrophoresis. In immunoprecipitation studies of ^3H -labeled platelets subjected to digestion with trypsin or with chymotrypsin, C-34 identified neither the glyocalicin nor the amino-terminal 45 kDa fragment of GPIb alpha that were immunoprecipitated by mab AS-2 or by mab AS-7.

Thus, using three independent techniques (immunoprecipitation of platelet glycoproteins following radiolabeling of intact platelets and subsequent proteolytic digestion of these glycoproteins; immunoprecipitation of radiolabeled purified glyocalicin; crossed immunoelectrophoresis of platelet glycoproteins) (Miller et al. 1990), it has been shown that while C-34 recognizes an epitope within the GPIb/IX complex, this epitope does not appear to reside within glyocalicin.

While these studies reported a relatively simple method that succeeded in epitope mapping mabs AS-2 and AS-7 to the 45 kDa region of GPIb alpha, this work demonstrated that mab C-34 cannot be mapped to any single tryptic or chymotryptic domain of glyocalicin.

Additionally, mab C-34 does not produce immunoprecipitation patterns similar to those of a mab recognizing GPIX.

5 Biopanning of Mab C-34 With Bacteriophage Display Libraries

Scott and Smith (1990) presented a method of defining peptide ligands by using randomly synthesized peptide inserts in bacteriophage. Related methods were
10 published by Cwirla et al. (1990) and by Devlin et al. (1990). Since that time a literature has arisen in which both the original hexapeptide inserts and larger inserts have been used in identifying epitopes recognized by monoclonal antibodies. This technique has great
15 potential for the detection of critical epitopes within the platelet vWF receptor known as GPIb/IX. The studies disclosed herein focus on monoclonal antibody C-34, but can be applied to other monoclonal antibodies having binding sites (epitopes) within GPIb/IX by the methods
20 disclosed herein for mab C-34.

A well-balanced decapeptide (10-mer) library from Dr. Bruce Malcom of Alberta, Canada (described by Christian et al. 1992) and a dodecapeptide (12-mer) library from Clontech Laboratories (Palo Alto, CA) were
25 used. In the dodecapeptide library, a reduced frequency of adenosines at the first two positions of each codon causes a characteristic underrepresentation of the following amino acids indicated by their one-letter codes: I, M, T, N, K, Y, H, Q, D, and E. The libraries have both
30 been constructed into a Fuse 5 vector (Scott and Smith 1990) by the insertion of a mixture of synthetic oligonucleotides, with the random decapeptides (or modified-random dodecapeptides) fused to the minor viral coat protein pIII of the bacteriophage. The libraries

each have a complexity of approximately 3×10^8 independent clones, and a titer of 10^{12} to 10^{14} per ml. While the Malcom library constitutes only a partial decapeptide library, it is complete as a hexapeptide library.

5 The strategy for using these libraries largely follows the review recently presented by Scott (1992) and employs, with modifications, the detailed methodology for use of this system as described recently by Smith and Scott (1993). The strategy used herein is as follows.

10 Specifically, in the first round of biopanning a 60 mm streptavidin-coated petri dish is filled with blocking solution (0.5% BSA, 0.1 M NaHCO_3 , 0.1 $\mu\text{g/ml}$ streptavidin, 0.2% NaN_3) for 2 hours, then washed three times with TBS-0.5% Tween. Next, 1 μl of the library
15 (about 1×10^{11} phage) that has been incubated overnight at 4°C with 1 μg of biotinylated Mab is diluted with 1 ml of TBS-Tween, and this mixture is then added to the petri dish and rocked for 15 minutes at room temperature. The petri dish is washed 10 times with TBS-Tween, and bound
20 phage is eluted by pipetting 800 μl of 0.1 N HCl (pH adjusted to 2.2 with glycine) - 1 mg/ml BSA into the dish. The eluate is then pipetted into a microfuge tube containing 48 μl of 2M Tris, to bring the pH up to about 8.

25 The eluate is concentrated and washed twice in TBS using an Amicon Centricon-30 filter (Amicon, Inc., Beverly, MA). This final product is titered out by making dilutions from a small amount of concentrated eluate in TBS-0.1% gelatin and adding 1 μl of each
30 dilution made to 19 μl of TBS-gelatin, then adding 20 μl of starved K91 *E. coli* cells and incubating for 10 minutes at room temperature. After adding 200 μl of NZY medium containing 0.2 $\mu\text{g/ml}$ tetracycline (Tc) and incubating at 37°C for 1 hour, the mixture is plated out

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on NZY agar plates containing 40 $\mu\text{g/ml}$ tetracycline and allowed to grow up overnight at 37°C.

After titering, the entire concentrated eluate from the first round of biopanning (about 50 μl) is added to an equal volume of fresh starved K91 cells, and amplification performed as described by Smith and Scott (1993). Following the first PEG/NaCl precipitation, the resulting pellet is dissolved in 1 ml TBS. Phage is then precipitated a second time with PEG/NaCl, allowed to stand at least 1 hour at 4°C, and the precipitate collected following centrifugation at 4°C. After careful removal of all the supernatant, the pellet is dissolved in 100 μl TBS. This amplified product can then be titered.

The first round of biopanning results in a yield of $5 \times 10^{-7}\%$. The second biopanning also used 1 μg of biotinylated C-34 with 1×10^{11} phage, resulting in a yield of $4 \times 10^{-3}\%$. The second round of biopanning is concentrated and amplified as in the first round. In the third round, 0.01 μg of biotinylated C-34 was biopanned against 2.5×10^{11} phage, with a resulting yield of $3 \times 10^{-4}\%$. The third round is stopped after eluting the bound phage from the petri dish. This eluate is not concentrated or amplified. Titerings are done before and after each round, and the percent yield is calculated as the number of bacteriophage obtained in an elution fraction relative to the initial number of bacteriophage (Christian et al. 1992). A yield should generally be greater than 10^{-5} to exceed background, with values of 10^{-4} to 10^{-1} typically observed. Increasing percent yields in subsequent rounds of biopanning are, in particular, suggestive that clones of increasing affinity are being selected.

For studies directed towards discovering a peptide binding the mimotope peptide (SEQ ID NO:1:

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AWNWRYREYV), two rounds of biopanning against the original decapeptide library were performed, using 1 μ g of biotinylated mimotope peptide in the first round and 0.01 μ g in the second round. Resulting yields were 3×10^{-5} % and 2×10^{-3} %, respectively.

In some experiments, an immunological screening assay, as described by Christian, et al. (1992) may be performed using NZY + Tc agar plates containing about 500 well-separated colonies. The colonies are transferred to nitrocellulose membrane filters (Biorad Laboratories, Hercules, CA), and the filters are immediately washed twice in TNT Buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20), blocked for 30 minutes at room temperature with gentle agitation in 20% normal goat serum in TNT buffer, then incubated for 2 hours at room temperature in primary mab that has been diluted 1:1000 in blocking buffer. The filters are washed sequentially for 10 minutes at room temperature each wash, in washing buffer A (TNT Buffer + 0.1% BSA), washing buffer B (TNT Buffer + 0.1% BSA + 0.1% NP-40), and then again washing buffer A, and incubated in a secondary peroxidase-conjugated goat anti-mouse IgG for 1-1/2 hours at room temperature. The filters are washed as before, then put in a final wash of TN (10 mM Tris, pH. 7.5, 150 mM NaCl). Color development is observed after putting filters in ABTS substrate.

Small cultures of individual colonies are then grown up overnight, by either: a) selecting the colonies that were positive from the immunological screening; or b) skipping the screening step and randomly selecting colonies (about 100). Each colony is inoculated into 2 ml of NZY medium containing 20 μ g/ml tetracycline, and these small cultures grown up overnight at 37°C, with vigorous shaking. The next day cultures are centrifuged

Following subsequent centrifugation and removal of 5 supernatant, the pellet is dissolved in 1 ml TBS.

15 Due to the quite GC-rich Sfi 1 cloning site
flanking the insertion region (Christian et al. 1992),
sequencing reactions are carried out using the Sequenase
7-deaza dGTP DNA sequencing kit (Amersham-US
Biochemicals, Arlington Heights, IL) with ³²P-dATP and an
20 antisense primer located approximately 40 nucleotides 3'
to the insert site (primer having SEQ ID NO:100: 5'
CTCATAGTTAGCGTAACG-3'). Samples are run on a standard 6%
sequencing gel using an IBI STS 45 sequencing apparatus
(Eastman Kodak Company, Rochester, NY).

25 The GCG software (Genetics Computer Group,
Inc., Madison WI) is helpful for aligning sequences
obtained from multiple clones in order to find consensus
sequences. Certainly in the case of new mabs for which
binding sites are sought, but even in the case of mab C-
30 34, there is an interest in searching for sequences not
only in GPIb alpha, but also in GPIb beta, GPIX, and in
fact other platelet proteins that have been deposited in
the available databases (Swiss Prot, Gen Bank, EMBL,
etc.). Indeed, this analysis may provide important new

information suggesting that a particular monoclonal antibody's epitope may be comprised of multiple components of the GPIb/IX complex that must accordingly be in close spatial proximity.

5 At this point, an ELISA assay can be used to evaluate individual clones, if the number of clones is high. In brief, phage having undergone two PEG precipitations, and subsequently adjusted for titer, can be incubated overnight with biotinylated mab, following
10 which the mab-phage mixture can be added to wells of microtiter plates that have been previously coated with formalin-fixed platelets (or other suitable immobilized target recognized by the mab). Following a series of washing steps, avidin-peroxidase is added, the wells
15 washed again, chromogenic substrate added, and the wells eventually read on an ELISA plate reader. The relative decrease in strength of signal in this assay provides guidance as to the most promising clones for further study. Consensus peptides identified in this manner can
20 be chemically synthesized and characterized with respect to ability to bind original antibody. Peptides showing high binding affinity for the antibody can then be used as immunogens in mice and/or rabbits.

25 Epitope Mapping Studies of mab C-34

 The two phage display libraries discussed above were employed in mapping studies with mab C-34. Results with the balanced, 10-mer peptide library were quite definitive with respect to strong consensus development
30 among clones selected after two or three rounds of biopanning. Not only is there an evident consensus towards the 9-mer sequence SEQ ID NO: 38: W N W R Y R E Y V, but the 10-mer peptide including this sequence (SEQ ID NO: 1) with an amino-terminal alanine appeared to have

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the greatest selective advantage in the biopanning, since clones bearing this sequence were found the most frequently.

The series of cloned sequences is included in 5 alignment form below. Double-underlines represent consensus amino acids and single-underlined amino acids represent significant homology to the consensus.

					<u>Frequency</u>
10	C34	Clone	SEQ ID NO:1:	. <u>A</u> <u>W</u> <u>N</u> <u>W</u> <u>R</u> <u>Y</u> <u>R</u> <u>E</u> <u>Y</u> <u>V</u>	52
	C34	Clone	SEQ ID NO:2:	. <u>K</u> <u>W</u> <u>N</u> <u>W</u> <u>R</u> <u>N</u> <u>K</u> <u>K</u> <u>Y</u> <u>V</u>	1
	C34	Clone	SEQ ID NO:3:	. <u>L</u> <u>S</u> <u>T</u> <u>W</u> <u>R</u> <u>Y</u> <u>F</u> <u>E</u> <u>Y</u> <u>V</u>	14
	C34	Clone	SEQ ID NO:4:	. <u>Y</u> <u>L</u> <u>G</u> <u>W</u> <u>R</u> <u>Y</u> <u>S</u> <u>E</u> <u>Y</u> <u>V</u>	7
	C34	Clone	SEQ ID NO:5:	. <u>T</u> <u>Q</u> <u>M</u> <u>W</u> <u>R</u> <u>A</u> <u>R</u> <u>E</u> <u>Y</u> <u>L</u>	2
15	C34	Clone	SEQ ID NO:6:	... <u>W</u> <u>R</u> <u>Q</u> <u>R</u> <u>E</u> <u>Y</u> <u>W</u> <u>D</u> <u>P</u> <u>V</u>	1
	C34	Clone	SEQ ID NO:7:	. <u>E</u> <u>G</u> <u>S</u> <u>W</u> <u>R</u> <u>Y</u> <u>R</u> <u>K</u> <u>G</u> <u>G</u>	1
	C34	Clone	SEQ ID NO:8:	<u>G</u> <u>Y</u> <u>H</u> <u>W</u> <u>R</u> <u>N</u> <u>W</u> <u>E</u> <u>Y</u>	2
	C34	Clone	SEQ ID NO:9:	<u>K</u> <u>G</u> <u>F</u> <u>L</u> <u>W</u> <u>R</u> <u>A</u> <u>R</u> <u>N</u> <u>W</u>	1
	C34	Clone	SEQ ID NO:10:	<u>M</u> <u>N</u> <u>W</u> <u>K</u> <u>H</u> <u>W</u> <u>R</u> <u>A</u> <u>R</u> <u>H</u> .	1
20	C34	Clone	SEQ ID NO:11:	<u>F</u> <u>K</u> <u>W</u> <u>R</u> <u>E</u> <u>W</u> <u>R</u> <u>G</u> <u>K</u> <u>L</u>	1
	C34	Clone	SEQ ID NO:12:	. <u>P</u> <u>D</u> <u>R</u> <u>Q</u> <u>V</u> <u>R</u> <u>L</u> <u>W</u> <u>V</u> <u>R</u>	1
	C34	Clone	SEQ ID NO:13:	<u>R</u> <u>V</u> <u>L</u> <u>R</u> <u>H</u> <u>W</u> <u>H</u> <u>P</u> <u>R</u> <u>T</u>	1
	C34	Clone	SEQ ID NO:14:	. <u>G</u> <u>R</u> <u>R</u> <u>V</u> <u>W</u> <u>M</u> <u>L</u> <u>N</u> <u>H</u> <u>G</u>	2
	C34	Clone	SEQ ID NO:15:	. <u>K</u> <u>K</u> <u>G</u> <u>R</u> <u>H</u> <u>H</u> <u>V</u> <u>T</u> <u>R</u> <u>V</u>	22
25	C34	Clone	SEQ ID NO:16:	. <u>G</u> <u>G</u> <u>V</u> <u>C</u> <u>K</u> <u>C</u> <u>W</u> <u>Q</u> <u>C</u> <u>L</u>	1
	C34	Clone	SEQ ID NO:17:	<u>F</u> <u>S</u> <u>H</u> <u>S</u> <u>Y</u> <u>G</u> <u>S</u> <u>A</u> <u>I</u> <u>R</u>	1
	C34	Clone	SEQ ID NO:18:	<u>M</u> <u>H</u> <u>G</u> <u>H</u> <u>R</u> <u>R</u> <u>P</u> <u>G</u> <u>L</u> <u>A</u>	1
	C34	Clone	SEQ ID NO:19:	<u>M</u> <u>S</u> <u>K</u> <u>K</u> <u>P</u> <u>H</u> <u>L</u> <u>G</u> <u>L</u> <u>R</u>	1
	C34	Clone	SEQ ID NO:20:	<u>T</u> <u>M</u> <u>W</u> <u>V</u> <u>E</u> <u>L</u> <u>Y</u> <u>S</u> <u>L</u> <u>K</u>	1
30	C34	Clone	SEQ ID NO:21:	<u>F</u> <u>V</u> <u>D</u> <u>P</u> <u>G</u> <u>R</u> <u>A</u> <u>G</u> <u>R</u> <u>G</u>	1
	C34	Clone	SEQ ID NO:66:	<u>K</u> <u>R</u> <u>A</u> <u>W</u> <u>W</u> <u>K</u> <u>Q</u> <u>K</u> <u>W</u> <u>V</u>	1

Results with the second peptide display library that is partially restricted in its amino acid repertoire

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revealed a series of clones which bind to C-34 without any appearance of the mimotope consensus sequence SEQ ID NO:38. The series of cloned sequences from the second library is included in alignment form below. SEQ ID 5 NO:22 is the native sequence of GPIb alpha from amino acid 484 to 499, and represents a possible natural epitope sequence revealed by the clones isolated from the second library. The ' represents potential chymotrypsin cleavage sites. As above, double-underlines represent 10 the possible native sequence (SEQ ID NO:22) within this second library and single-underlined amino acids represent significant homology to the possible native sequence.

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C34b series versus GPIb 484-499

SEQ ID NO:22:		<u>C C L L P L G F Y V L G L F W L</u>
SEQ ID NO:23:	<u>F R C C V F S C C L L S</u>	
SEQ ID NO:24:	<u>G F R C L Y S L G G C F</u>	
SEQ ID NO:25:	<u>Y S L W G L P Y G D V V</u>	
SEQ ID NO:26:	<u>L P L I M F N G A G F F</u>	
SEQ ID NO:27:	<u>V W G L F R G L E N G S</u>	
SEQ ID NO:28:	<u>S L W R Q W R G L F V V</u>	
SEQ ID NO:29:	<u>T L S L F G G R D K G F</u>	
SEQ ID NO:30:	<u>I G P A Y S C L F R V C</u>	
SEQ ID NO:31:	<u>M S L F P L S F C R L I</u>	
SEQ ID NO:32:	<u>A L F S S V W G D V T L</u>	
SEQ ID NO:33:	<u>G W F G P F W Y R G S G</u>	
SEQ ID NO:34:	<u>F W Y S V G G V E G V V</u>	
SEQ ID NO:35:	<u>L G A F G G A G F L W R</u>	
SEQ ID NO:36:	<u>C R G I V F L F Y G W L</u>	
SEQ ID NO:37:	<u>F W L V K G A G A W R F</u>	

' = Potential Chymotrypsin Cleavage Site

The following cloned sequences were also obtained from the second peptide display library:

SEQ ID NO:39: QVRLWARAGAGQ
5 SEQ ID NO:40: GLAVTFGSVLEG
SEQ ID NO:41: VRWMCVIRLGVR
SEQ ID NO:42: RLWGPGVSRPVL
SEQ ID NO:43: CGSSLFRGPRCP
SEQ ID NO:44: LGISSLFLQLR
10 SEQ ID NO:45: TWGWDGVSYLFL
SEQ ID NO:46: TRSLFDDFVSLR
SEQ ID NO:47: CYASLFRSRLCA
SEQ ID NO:48: DGSVRVVWRLL
SEQ ID NO:49: LSGFPVALVRFA
15 SEQ ID NO:50: LGGGLLVGSVFP
SEQ ID NO:51: VWARGVFRDRFF
SEQ ID NO:52: TGLLAGPVWRWT
SEQ ID NO:53: WLGGIFSCLVCG
SEQ ID NO:54: WFLRDVGC GSCL
20 SEQ ID NO:55: SRCGVFTWC SRS
SEQ ID NO:56: RCLVGYRCWGGV
SEQ ID NO:57: GFRCLVMGGGCA
SEQ ID NO:58: CGFDLVCARLFG
SEQ ID NO:59: DSGVRWFFGFLG
25 SEQ ID NO:60: ILDGCFFLGRCP
SEQ ID NO:61: CVRWLVSAGCSG
SEQ ID NO:62: CVGCWLVC DVLL
SEQ ID NO:63: CLFVFAAGFACG
SEQ ID NO:64: SCALFGSCFGIS
30 SEQ ID NO:65: CWGGVGVCGLLV
SEQ ID NO:67: CVGGVASRCGVL
SEQ ID NO:68: SGAVLAGPFGVW
SEQ ID NO:69: CRAFTRVGVCVW
SEQ ID NO:70: RCLVGYVVGGVW

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SEQ ID NO:71: VCLVYRSVDCWA
SEQ ID NO:72: WRVVFVFTCVVWA
SEQ ID NO:73: LWREWRGLFAVL
SEQ ID NO:74: SGAVLAGPLWRL
5 SEQ ID NO:75: FVVRGGTFLFVR

SEQ ID NO:77: TGLLAGPVWRWT
SEQ ID NO:78: DSGVRWFFGFLG
SEQ ID NO:79: CAWHRLSFCGLV
10 SEQ ID NO:80: CFGSALVLAVLA and
SEQ ID NO:81: WFWDMSGEWGGL.

Comparison of Consensus Sequence to Native Sequences

Considerable effort was extended in trying to
15 relate the consensus sequence of the above peptide (SEQ
ID NO:38) to native sequences within GPIb alpha or other
known proteins in the Swiss Protein or NCBI data banks.
No such relation was found. This sequence accordingly
represents a "mimotope" - i.e., a peptide which mimics a
20 native epitope (a binding site for a monoclonal
antibody), despite a lack of apparent homology at the
primary amino acid sequence level (for mimotopes, see:
Motti et al. 1994, Larocca et al. 1992, Lenstra et al.
1992, Balass et al. 1993, Hobart et al. 1993, and Luzzago
25 et al. 1993). As noted after reviewing SEQ ID NOs: 1-21
and 66 above, not all selected clones appear to be part
of this consensus group, and it is possible that with
further sequencing clues as to the native epitope may be
derived.

30 By using the second peptide display library
that is partially restricted in its amino acid
repertoire, another series of clones ("C34b" series)
binding to C-34 without appearance of the mimotope
consensus peptides were obtained. Following sequencing

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of these clones, a FASTA analysis (Pearson and Lipman 1988; Pearson 1990) was performed upon this group of clones by moving a 7-amino acid window along the sequence of GPIb alpha, advancing one amino acid at a time, and
5 determining the group score as a function of position in the GPIb alpha molecule.

The results do not, in general, offer compelling matches in the sense of consensus development among the clones. However, the possible native GPIb
10 alpha sequence revealed by this analysis is represented by SEQ ID NO:22.

Aggregation Studies

Citrated human platelet-rich plasma (PRP) was
15 prepared by standard methods (Miller et al. 1983). For study of C-34 neutralization by mimotope peptide, 350 μ L of PRP containing 150,000 platelets/ μ L was incubated for 10 min at 22°C with phosphate-buffered saline (PBS), 20 μ g/mL C-34 mab, or 20 μ g/mL C-34 that had previously been
20 incubated for 30 min at 22°C with varying concentrations of peptides. The PRP was then brought to 37°C and stirred at 1200 rpm in a Chrono-Log lumi-aggregometer (Chrono-Log Corporation, Havertown, PA). Aggregation was initiated by the addition of 1 mg/mL ristocetin (Helena
25 Laboratories, Beaumont, TX). For screening of bacteriophage clones displaying potential anti-mimotope peptides, 150 μ l of PEG/NaCl precipitated phage was incubated with 250 μ l of citrated PRP for one hour at 22°C, transferred to the aggregometer, following which
30 ristocetin was added at a final concentration of 0.8 mg/ml. Study of the inhibitory potency of synthetic peptides upon vWF-dependent platelet aggregation was performed by pre-incubating 150 μ L of varying dilutions of peptide dissolved in PBS, pH 6.0 for 2-4 hr at 22°C

with 250 μ L of formalin-fixed (Macfarlane et al. 1975) platelets (1.5×10^5 /mL), following which the mixture was warmed to 37°C in the aggregometer, purified vWF (Miller et al. 1983) (1 U/mL) was added, and aggregation was initiated by the addition of 0.9 mg/mL ristocetin.

Synthesized Peptide

A peptide including the consensus sequence (SEQ ID NO: 38) was chemically synthesized (Genosys Biotechnologies, The Woodlands, Texas). The synthesized peptide had an amino acid sequence corresponding to SEQ ID NO:1: AWNWRYREYV. A modification of this peptide with a biotin attached to the amino-terminal alanine (N-hydroxysuccinimide hexanoic acid long chain spacer arm biotinylation) was also synthesized. One mg of the chemically synthesized biotinylated peptide was dissolved in one ml of water containing 20 μ l of DMSO. Since C-34 at a final concentration of 20 μ g/mL is a potent inhibitor of ristocetin-induced aggregation in citrated platelet-rich plasma (PRP), the synthetic peptide's potency was assessed by examining whether the peptide could neutralize the inhibitory activity of C-34 in this setting. Accordingly, approximately 10 μ g of C-34 was incubated at 22°C for 30 minutes with varying concentrations of test or control peptide, following which the mixture was added to PRP in a final volume of approximately 0.5 ml for an additional 10 minutes at 22°C. As can be seen from the resulting aggregation curves (Figures 1-7), the synthesized peptide fully neutralized the C-34, producing half-maximal neutralization of the C-34 at about 1.0 μ g/ml, which is approximately 0.55 μ M for the biotinylated peptide. A similar pattern of C-34 antibody neutralization was observed when the non-biotinylated form of the peptide

(having SEQ ID NO:38) was used, with half-maximal neutralization at approximately 3.0 μ M. The peptide (native or biotinylated) by itself did not induce platelet aggregation, nor did it appear to have non-specific effects, inasmuch as it had no influence on ADP-induced aggregation.

More specifically, Fig. 1 shows the ristocetin-induced full aggregation of platelets in the presence of von Willebrand factor. Fig. 2 shows the inhibition of ristocetin-induced aggregation of platelets by 20 μ g/ml of mab C-34. Figs. 3-7 show varying degrees of neutralization of the inhibition of ristocetin-induced aggregation of platelets by 20 μ g/ml of mab C-34 in the presence of 0.14, 0.27, 0.55, 1.1, and 2.3 μ M of the synthetic biotinylated peptide mimotope having SEQ ID NO:1, respectively. In Fig. 3, 0.14 μ M of the peptide does not neutralize the C-34 inhibition; in Fig. 7, 2.3 μ M of the peptide fully neutralizes the C-34 inhibition, and Figs. 4-6 show varying degrees of neutralization of the C-34 inhibition.

Additional Use of Synthesized Peptide

The chemically synthesized peptide can be conjugated to bovine serum albumin and used for raising polyclonal antibodies in rabbits. Standard procedures can be used to immunize the rabbits and to collect serum, as described below. Polyclonal antibody can be tested for its ability to bind to normal platelets, as well as to the wild-type and valine 233 mutant forms of recombinant GPIb alpha. For polyclonal antibody that shows a high affinity binding to platelets, functional studies can then be undertaken. These studies include adhesion, aggregation, agglutination, and vWF binding. F(ab)'₂ and Fab fragments of the polyclonal antibody can

be made if steric hindrance appears to be preventing an accurate evaluation of more specific modulating effects of the antibody (Becker and Miller 1989, Kupinski and Miller 1986, and Miller et al. 1986). Polyclonal
5 antibody to the synthetic peptide that recognizes or stabilizes a conformation associated with heightened or diminished affinity for binding vWF can be obtained at \geq 95% purity and conjugated to bovine serum albumin or to another carrier protein, for the production of murine
10 monoclonal antibodies.

Production of Antibodies to Synthesized Peptides

Mice: Monoclonal antibody production can be carried out using BALB/c mice. Immunization of the B-
15 cell donor mice can involve immunizing them with antigens mixed in TiterMax™ adjuvant as follows: 50 μ g antigen/20 μ l emulsion x 2 injections given by an intramuscular injection in each hind flank on day 1. Blood samples can be drawn by tail bleeds on days 28 and 56 to check the
20 titers by ELISA assay. At peak titer (usually day 56) the mice can be subjected to euthanasia by CO₂ inhalation, after which splenectomies can be performed and spleen cells harvested for the preparation of hybridomas by standard methods.

25 Rabbits: Polyclonal antibodies can be raised in New Zealand white rabbits. Preimmune serum can be collected from rabbits sedated with ketamine/rompun (ketamine HCl at 20 mg/kg IM and xylazine HCl at 4 mg/kg IM) via the auricular artery. Ten to fifteen percent of
30 the total blood volume can be collected at each bleeding. The hair over the ear can be shaved with a #40 clipper blade, wiped with 70% alcohol, and a sterile 22 gauge butterfly can be used for blood collection. The antigen can be mixed with either RIBI adjuvant or TITER-MAX™

adjuvant and used according to the manufacturer's instructions. The back can then be shaved, wiped with 70% alcohol, and a sterile 25 gauge needle with the antigen/adjuvant mixture therein can be used to
5 administer subcutaneously and intramuscularly as recommended by the manufacturer's instructions. Immune serum samples can be collected as described for preimmune samples. When sufficient titers are reached, the animal can be anesthetized with sodium pentobarbital (60 mg/kg
10 BW) via the lateral ear vein until deep anesthesia is achieved. Blood can be immediately collected via cardiac puncture into plastic centrifuge tubes and allowed to clot; afterwards, the blood can be centrifuged and the serum aspirated and frozen at -70° C. For euthanasia,
15 while under sodium pentobarbital anesthesia at a dosage of 60 mg/kg, the rabbit can be exsanguinated via cardiac puncture.

Development of C-34 Anti-Mimotope Peptides

20 The mimotope decapeptide itself was then used as a probe to search for "anti-mimotope" peptides. Specifically, while a number of peptides might interact with some portion of the mimotope peptide exposed in solution, an "anti-mimotope" peptide would be defined as
25 one that was not only selected in multiple rounds of biopanning, but that also provided some measure of functional interaction with the native epitope, thereby resembling the original monoclonal antibody. As shown in Fig. 8, one single clone of 46 bacteriophage clones
30 purified and sequentially tested demonstrated inhibitory activity above background level in a functional platelet assay. This "anti-mimotope" clone displayed the sequence having SEQ ID NO:94: RHVAWWRQGV-the carboxyl terminal half of which is identical to residues 230-234 of GPIb

alpha, with only the conservative (Lys-Arg) substitution at residue 231. (See GPIb alpha sequence from 225-237 [SEQ ID NO:101] and GPIb alpha sequence from 225-234 [SEQ ID NO:173: ENVYVWKQGV]). Of the 57 unique sequences ultimately determined, 5 additional sequences showed varying degrees of structural homology as shown below. Additional anti-mimotope sequences also included the following:

- 10 SEQ ID NO:157: AYGVRHLGLS
SEQ ID NO:158: KKWGQHRQRS
SEQ ID NO:159: WRWMHWMPHA
SEQ ID NO:160: WHWLARHRTV
SEQ ID NO:161: RHRHRGFQPR
15 SEQ ID NO:162: RGWRWHKYWQ
SEQ ID NO:163: KRHAWMKSRL
SEQ ID NO:164: LLLVGGSELT
SEQ ID NO:165: KKVWMFSYNE
SEQ ID NO:166: LSCRCRAFV
20 SEQ ID NO:167: HEGCEAQDEL
SEQ ID NO:168: SVRHIWFHVK
SEQ ID NO:169: GTWDLWRKGS
SEQ ID NO:170: RWLWPRVHKT
SEQ ID NO:171: HSPFRHVQPR and
25 SEQ ID NO:172: WVRGHHREVR.

661060: 4788920

SEQ ID NO:101:

GPIb α 225-237 EN V Y V W K O G V D V K

SEQ ID NO:94:

R H V A W W R O G V

SEQ ID NO:95:

A K H R W W R P V

SEQ ID NO:96:

K H F M R H R H G V

SEQ ID NO:97:

A G L N H W W K H K

SEQ ID NO:98:

R R S T W H W H A

SEQ ID NO:99:

V A K W R H W N R Q*

Further studies were undertaken with chemically synthesized peptide having SEQ ID NO:94: RHVAWWRQGV. This decapeptide was able to inhibit ristocetin-induced aggregation fully, with an IC_{50} occurring between 200-400 $\mu\text{g/mL}$ (Fig. 9). A (Gly-Val) substitution at position 9 (SEQ ID NO:104), corresponding to the mutation observed in PT-vWD, slightly lowered the IC_{50} , although nearly full inhibition was again seen by 715 $\mu\text{g/mL}$. In order to approximate more closely the native structure, peptides with an (Arg-Lys) substitution at position 7 were then studied. As shown in Fig. 10, a more dramatic difference between the Gly and the Val forms of the Lys-containing peptides was observed. Whereas the RHVAWWKQVV (SEQ ID NO:105) peptide retained potent inhibitory activity, the RHVAWWKQGV (SEQ ID NO:106) peptide was unable to exert more than slight inhibition, except at the highest concentrations tested. Finally, both the wild-type GPIb alpha 228-237 peptide (SEQ ID NO:108) containing Gly at residue 233 and the PT-vWD variant with Val replacing Gly at this position (SEQ ID NO:107) were synthesized. As shown in Fig. 11, the wild-type peptide was virtually without inhibitory activity. In contrast, the peptide corresponding to the PT-vWD mutant was capable of fully inhibiting ristocetin-induced aggregation, with an IC_{50} of approximately 400 $\mu\text{g/mL}$. Lyophilized peptides were reconstituted in PBS, pH 6.0 and 150 μL of varying dilutions incubated for 2-4 hr at 22°C with 250 μL of formalin-fixed platelets ($1.5 \times 10^5/\text{mL}$), prior to aggregometry in which the addition of 1 U/mL purified vWF was followed by the addition of 0.9 mg/mL ristocetin.

Three-Dimensional Description of Mimotope/Anti-Mimotope

Figs. 12a-12c show the proposed three-dimensional description of mimotopes and anti-mimotopes. In Fig. 12a, the region within the extracellular domain of platelet glycoprotein Ib alpha containing the original epitope 10 capable of recognizing monoclonal antibody C-34 is shown. Fig. 12b shows the structure of the mimotope peptide 12 which mimics the original epitope (10, as shown in Fig. 12a) in three-dimensional space, without sharing the primary amino acid sequence of the original epitope. The mimotope peptide 12 also recognizes, or binds to, monoclonal antibody C-34.

Fig. 12c illustrates the structure of the mimotope peptide 12 in relation to the structure of the anti-mimotope peptide 14. The anti-mimotope peptide sequence is complementary to the face of the mimotope peptide in three-dimensional space, as monoclonal antibody C-34 was to the original epitope (see Fig. 12a).

Epitope Mapping Studies of mab SZ-2

Epitope mapping studies were also conducted using monoclonal antibody SZ-2. The choice of mab SZ-2 (Ruan et al. 1987) was made because its epitope is known to lie within the 45 kDa region of GPIb alpha (Fox et al. 1988; Molino et al. 1993); the epitope is likely to be relatively conformation-independent since SZ-2 blots strongly to GPIb alpha, glyocalicin or GPIb alpha 45kDa fragment that has been denatured in SDS prior to transfer to nitrocellulose (Molino et al. 1993); and there may be widespread interest in epitope localization of this mab since it is available commercially and appears to be being used in a wide variety of investigative and clinical studies worldwide.

The well-balanced, 10-mer random peptide display library was used with SZ-2. Following either two or three rounds of biopanning with immunoscreening in the third round, bacteriophage clones were sequenced and the 5 resulting predicted peptide sequences were analyzed for convergence upon a clear-cut pattern that hopefully is contained within the first ~300 amino acids of the mature GPIb alpha molecule. The resulting displayed sequences were compared with the available set of glycoprotein 10 sequences known to exist on the platelet surface, including GPIa, GPIb alpha, GPIb β , GPIIb, GPIIIa, GPIV, GPIX, and the platelet FCgamma₂ receptor.

The most convincing correspondence of multiple phage sequences with a natural platelet sequence may be 15 with residues of the platelet FCgamma₂ receptor rather than of GPIb alpha, based upon the following observations: First, while GCG FASTA and WORDSEARCH analyses of phage sequences compared with residues 1-300 of GPIb alpha do show several favored regions of 20 similarity, there is not yet a single, short stretch of amino acids in the native molecule that emerges in a convincing fashion as an obvious match. Second, using the first 50 clones for which highly purified PEG precipitates were prepared and titered, ELISA assays were 25 performed in which the binding of phage to biotinylated SZ-2 inhibits the subsequent binding of the SZ-2 to immobilized glyocalicin. Only one of the 50 clones, displaying the sequence having SEQ ID NO:83: W H W R S S W K S G, proved capable of fully neutralizing SZ-2, and 30 no other clone then available came even close in neutralizing potency. This clone, however, did not appear to represent an evident convergent pattern of the series of clones, nor did it provide a more extensive match to sequences within GPIb alpha than other clones

5
10

FCGB_HUMAN 148 I V L R C H S W K D K P L V K
SEQ ID NO:102:

SEQ ID NO:84: H R P L S W K G R A
SEQ ID NO:83: W H W R S S W K S G
SEQ ID NO:85: W H R R P M S W Y S
SEQ ID NO:86: A R I K I W K P R W
SEQ ID NO:87: K R G W H W K S L H
SEQ ID NO:88: K K S W W V R M P R
SEQ ID NO:89: A K S W R Y W R M P
SEQ ID NO:90: K R W K V Y H R W P
SEQ ID NO:91: L H R W K Q S P R T
SEQ ID NO:92: L I R W K P H G W R
SEQ ID NO:93: Q K K F F S R W K H

GPIbα 221 D N A E N V Y V W K Q G V D V K A M T
SEQ ID NO:103:

SEQ ID NO:91: L H R W K Q S P R T
SEQ ID NO:83: W H W R S S W K S G

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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